INHIBITION OF NEWCASTLE DISEASE VIRUS INFECTION OF CHICKEN EMBRYO CELLS BY CAFFEINE

by

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SECTION I

INTRODUCTION

INTRODUCTION

Newcastle disease is a respiratory disease of chickens of considerable importance to the United States poultry industry. For this reason, much research has been accomplished to determine the *in vivo* and *in vitro* effects caused by Newcastle disease virus (NDV) infection in an effort to understand the mechanism(s) involved.

NDV, a paramyxovirus, is a roughly spherical, enveloped virus containing single-stranded ribonucleic acid (RNA). Some paramyxoviruses are known to infect humans. NDV is an ideal model system for the study of these viruses. The use of drugs to study paramyxoviruses may give information concerning growth control mechanisms. It is hoped that one day this information may lead to the development of viral specific drugs which have minimum toxic effects on the virus host cells. Also, there is always the possibility that information gained may help in understanding cellular processes in general.

Caffeine is a drug that has been used in studies involving prokaryotic and eukaryotic cell systems. Some investigators have utilized caffeine to examine UV survival and induction processes of DNA viruses. However, minimal investigation has been done with RNA viruses. The purpose of this investigation was to describe the effects of caffeine on NDV synthesis in cultured chick embryo cells, and to elucidate a possible mechanism for the effects observed.

Caffeine was found to inhibit synthesis of viral components including RNA and protein and to significantly reduce the number of progeny formed. The progeny which were recovered from NDV infected cells appeared extremely

sensitive to ultraviolet light and to freezing and thawing. To determine a possible explanation for this, the effect of caffeine on the polypeptide composition of the purified virions was examined.

This thesis has been written in the form of a manuscript which was prepared according to the guidelines for publication in Infection and Immunity. The manuscript "Inhibition of Newcastle Disease Virus Infection of Chicken Embryo Cells by Caffeine" has been submitted for publication in Infection and Immunity. An additional section giving a historical review of the literature and the literature cited has been included.

SECTION II

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SECTION III

LITERATURE REVIEW

LITERATURE REVIEW

History and pathogenicity

Newcastle disease was first observed in chickens on the island of Java (53). A similar disease which was viral in nature was next recorded in Newcastle-on-Tyne, England from which the name "Newcastle disease virus" was derived (25). After outbreaks occurred in California (4), it was considered a major endemic disease of the United States poultry industry. To eradicate the disease, infected chicken flocks must often be slaughtered. However, there is available vaccines of inactivated or attenuated NDV.

Newcastle disease virus (NDV) is transmitted by ingestion of contaminated drinking water and possibly by inhalation of airborne particles (51). There is a wide spectrum of virulence for chickens which may be symptomless or fatal. Infection usually occurs in the respiratory tract, but may also affect the nervous system (penumoencephalitis) and the vicera.

NDV infection in man is usually apparent as an acute conjunctivitis. It was first observed as the result of a laboratory accident (11), and is sometimes found in poultry workers and veterinarians exposed to diseased birds.

Related paramyxoviruses

Newcastle disease virus is a paramyxovirus. The paramyxovirus group is a large one which includes the parainfluenza viruses types 1-5, Newcastle disease, mumps, Yucaipa and Nariva viruses. Also, measles, canine distemper and rinderpest viruses are usually included in the group. Paramyxoviruses which are known to infect man cause mumps, measles and subacute sclerosing panencephalitis (40). They are also associated with

multiple sclerosis (6) and systemic lupus erythematosis (34, 52). Paramyxoviruses have been reviewed by Robinson and Duesberg (68), Blair and Duesberg (7), Compans and Choppin (20), Kingsbury (47, 49) and Choppin and Compans (16). Much of the knowledge of the structure and replication of paramyxoviruses has been derived from studies on NDV.

NDV ribonucleic acid

The virions of paramyxoviruses are among the largest RNA virions. Most are spherical in shape and range in diameter from 120 to 200 nm (32). The virions consist of a membrane-containing envelope covered with surface protein projections or spikes. This envelope encloses the ribonucleoprotein which is a large helical nucleocapsid with the genome a single-stranded, continuous RNA molecule (7).

The RNA of Newcastle disease virus was the first genome of the paramyxovirus shown to be of high molecular weight as determined on the basis of a sedimentation coefficient of 50-57S in sucrose gradients (2, 7, 26, 45, 65, 78). A sedimentation value of 50S is generally used following the accepted data of Kingsbury (48). The molecular weight of NDV-RNA has been estimated to be about 6.0×10^6 daltons on the basis of sedimentation under denaturing conditions (dimethyl sulfoxide) (27, 52). The base composition of NDV-RNA shows a relatively high content of uracil (26, 45). In some strains of NDV, the RNA appears capable of self-annealing as much as 30%, becoming ribonuclease resistant (69).

NDV polypeptides and lipids

The proteins of paramyxovirus are often named according to the convention of Scheid and Choppin (74), although differences are noted among the members of the group and even among their individual strains. Each

paramyxovirus virion has a nucleocapsid protein (NP) with a molecular weight of around 56,000-61,000 daltons. Paramyxovirus nucleocapsids are flexible, helical structures containing approximately 5% RNA (19, 46, 84). This flexibility is essential to enclosure of the structure within the virion. Part of the nucleocapsid protein is very resistant to proteolytic cleavage, while part is susceptible. The cleaved portion possibly confers flexibility upon the nucleocapsid, and it has been suggested that it may be involved in the recognition by the nucleocapsid of specific areas of membrane during maturation (64).

There appear to be at least two glycoprotein species; the larger one (HN) has a molecular weight of 67,000-74,000 daltons and is responsible for neuraminidase and hemagglutinating activities (73). The smaller glycoprotein (F), which contains hemolysis and cell fusion activities, has a molecular weight of about 53,000-56,000 daltons. It (F) is derived by proteolytic cleavage from a precursor protein (F_o) which is estimated to be 65,000 daltons. These molecular weight estimates are based on migration in SDS polyacrylamide gels, which are not exact for glycoproteins. The proteolytic cleavage appears to be host-dependent, as cleavage of Sendai virus occurs in chick embryo cells, but does not occur in MDBK (74) or L cells (38).

The two glycoprotein species form the spikes on the surface of the virion. These spikes are surface projections 8-12 nm in length (14, 39, 70). They have shallow penetration into the lipid bilayer and can be removed by treatment with proteolytic enzymes, while the other virion proteins are protected from the enzyme by the lipid bilayer (12, 13, 60). The spikes do not appear to play a significant structural role in maintaining the integrity of the viral membrane (16).

Associated with the envelope of paramyxoviruses is a nonglycosylated membrane protein (M) with a molecular weight of 38,000-41,000. Available evidence suggests that the M protein is located just beneath the lipid bilayer in paramyxoviruses, rhabdoviruses and myxoviruses (21). Interaction between this protein and the lipid of the membrane may play a role in maintaining the structure and integrity of the viral envelope. It may also function in the mechanism for excluding host protein from cellular membrane areas destined to become viral membrane (15). Finally, this protein may function during virus assembly as the site which is recognized by the viral nucleocapsid when it aligns under those areas of cell membrane which contain viral proteins and which become viral membrane (16).

A large molecular weight protein (L) band has been found near the origin of polyacrylamide gels of NDV and Sendai virus proteins by several investigators (10, 54, 89), and a possible transcriptase role has been suggested (36). However, this material is not always present nor present in the same amounts even from different preparations of the same strain of virus, and may simply represent an aggregate rather than a primary gene product. A variety of other minor protein species have been identified in different members of the paramyxoviruses.

Paramyxovirus proteins are not found in equimolar amounts in infected cells (37, 80) nor in purified virions. It appears that the abundance of NDV-mRNAs control the abundance of the polypeptides for which they are assumed to code (8).

The lipids of the paramyxovirus are present in a bilayer. Treatment with lipid solvents will destroy the infectivity and the structural integrity of the virion. Studies indicate that the structural rigidity of the bilayer depends on the lipid composition and not on differences in membrane-associated

proteins. Also, the spike glycoproteins neither penetrate deeply into the bilayer nor contribute significantly to the structure of the lipid phase (55). While virion protein composition appears to be virus dependent, the lipid composition of paramyxoviruses seems to be dependent upon the host cell (50, 55).

NDV infection of cultured cells

Most strains of NDV grow readily and produce cytopathic effects in all tissues of chick embryos and chick embryo tissue culture. A high yield of homogeneous NDV can be harvested from the allantoic fluid of chick embryos, while a much lower yield and a pleomorphic population of NDV particles are obtained from chick embryo cell tissue culture (62).

The sequence of events which occur during the infection of animal cells by RNA viruses include adsorption, penetration, uncoating, synthesis of viral components and assembly of progeny virus (85). NDV attach to specific receptor sites on chick embryo cells by electrostatic forces and hydrogen bonding (58). The cell receptor site consists of a mucoprotein containing N-acetylneuraminic acid (35). It is believed that adsorption to the cell receptor involves the virion hemagglutinin glycoprotein. The virion possesses neuraminidase which is capable of destroying these receptors during the release by budding. Although there were earlier conflicting reports, recent evidence indicates that the viral neuraminidase and hemagglutinating activities lie in the same protein (HN), although the existence of one or two active sites for the two activities has not been established (73, 74, 81).

The mechanism by which the virion genome enters the cell was believed to be by viropexis which is a phagocytic invagination (29, 77), although

fusion of the viral membrane with the cell membrane and penetration of the nucleocapsid alone has been proposed (1, 61). The conclusions of all these studies depend on the interpretation of electron micrographs.

The period between penetration of the virus and the appearance of new progeny virus is known as the eclipse or latent period. Virions of paramyxoviruses contain an RNA polymerase which transcribes the genome RNA in infected cells into 18, 22 and 35S mRNA molecules which are complementary in base sequence to the viral genome (9, 18). Both the Newcastle disease virus and Sendai virus RNA polymerase will synthesize predominantly single-stranded complementary 16-18S molecules in vitro (41, 79). This transcription is not sensitive to actinomycin D. The complementary RNAs are the messenger RNAs which direct the synthesis of paramyxovirion proteins (48, 63).

Most evidence indicates that viral transcription and replication occur in the cytoplasm of infected cells (9, 66, 86). Paramyxovirus replication requires neither cellular DNA synthesis nor transcription, and actinomycin D is often used in paramyxovirus infection to inhibit cellular macromolecular synthesis, thus making viral directed synthesis more visible (21).

The virus is assembled by budding at the cellular membrane. The nucleocapsid associates with areas of the membrane containing viral proteins (3, 31). The membrane envelope surrounds the nucleocapsid and is pinched off. It is currently believed that neuraminidase may play a role in actual release from the cell surface receptors.

Caffeine

The mutagenic effects of caffeine were independently discovered more than 25 years ago by Witkin in the United States and by Fries and Kihlman in Sweden. Since then, caffeine has been studied in bacterial, viral and various eukaryotic cell systems (43).

Caffeine (1,3,7-trimethylxanthine) was first discovered to produce point mutations in the conidia of the fungus Ophiostoma multiannulatum which led to an increased frequency of physiological mutants, i.e., mutants unable to synthesize some factor necessary for normal growth (33). Other initial studies found that caffeine and other methylated oxypurines were effective mutagens in growing cultures of Escherichia coli and T5 bacteriophage. However, they seem to be active only in the presence of oxygen and DNA synthesis. Also, caffeine mutagenesis in E. coli can be suppressed by addition of guanosine and adenosine to the growth medium.

The chromosome-breaking effects of methylated oxypurines involved fragmentation of chromatids in cultured mammalian cells exposed to caffeine in the S-phase (5, 42, 56). Possibly, lesions can be induced prior to the S phase, but the aberration is not realized until the affected chromosomes are replicated. The mutagenic effects appear to be somewhat temperature dependent with the maximum effect produced at about 23°C and rapidly decreasing as the growth temperature is lowered or raised (43). Also, the aberration frequency is reduced when ATP levels are lowered and is increased when pretreatment with adenine leads to high ATP levels.

Caffeine appears to bind to DNA by hydrophobic forces and by intercalation between the purine and pyrimidine bases (82). Also, investigators have shown that the affinity constant of caffeine for DNA is much higher for denatured than for native DNA (83). In addition, Domon et al., observed that binding of caffeine to DNA increases when DNA is partially denatured by ultraviolet light (23). Also, nuclease S_1, which preferentially digests denatured DNA, was shown to digest native DNA that had been treated with caffeine. Caffeine has been shown to affect the thermal stability of DNA, resulting in a melting profile characterized by two transitions. The first

transition, which was below the normal melting point of DNA, may have been the result of local unwinding of the DNA caused by caffeine (43).

Most of the research involving caffeine as a mutagen has been performed to examine its synergistic effect with another mutagen such as ultraviolet irradiation. Caffeine was shown to increase lethal and mutagenic effects of UV light on E. coli (59, 75, 87). This "mutational synergism" was interpreted to be a consequence of inhibition by caffeine of enzymatic dark repair of premutational lesions in DNA (16, 59, 88). This synergism was not seen in excision-defective (Hcr^-) strains of E. coli (17, 72, 76). It is concluded that caffeine enhances UV effects by decreasing the number of UV photoproducts that can be removed from the DNA by excision. Of a series of purine analogs tested, only methylated derivatives exhibited the synergistic effect with caffeine being the most effective (24).

In addition, caffeine may inhibit the photoenzymatic repair of lesions in E. coli DNA by competing with the photoreactivating enzyme for binding sites near the dimers (36). Whether caffeine acts by competing with the substrate for the active site on the enzyme or by competing with the enzyme for the substrate is still a matter of dispute.

Host cell reactivation by some means of repair has also been demonstrated in bacterial and animal virus systems (22, 30, 71, 72, 90). Caffeine was shown to inhibit "host cell reactivation" of bacteriophage by inhibiting excision of dimers. In yeast cells and some higher plant and cultured mammalian cells, caffeine is believed to potentiate cell mutagenesis and death by affecting post replication repair rather than excision repair (28, 44, 57, 67).

SECTION IV

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LITERATURE CITED

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SECTION V

MANUSCRIPT: INHIBITION OF NEWCASTLE DISEASE VIRUS INFECTION OF CHICKEN EMBRYO CELLS BY CAFFEINE

A study was undertaken to examine the effects of caffeine on Newcastle disease virus (NDV) infection of chicken embryonated cells. Addition of 10 mM caffeine (Caf+) to the growth medium produced a 95% reduction in progeny synthesis, a 63% reduction in RNA synthesis, a 45% reduction in protein synthesis, and 25% reduction in hemadsorption ability in NDV infected cultures when compared to untreated (Caf-), infected cultures.

Purified NDV obtained from caffeine-treated, infected cultures (Caf+) was more sensitive to ultraviolet light irradiation and to damage by freezing and thawing than was observed in Caf- virus. SDS-polyacrylamide gel electrophoresis revealed that purified virions (Caf+, Caf-) contained the same complement of polypeptides, but there was a significant variation in the quantities of several of the NDV polypeptides.

INTRODUCTION

Caffeine is a methylated purine analog which has been studied extensively using various cell systems and using cells infected with DNA viruses (10). Caffeine was an important tool in elucidating the DNA repair mechanisms in ultraviolet irradiated cells (10, 25). This drug has been used often in conjunction with ultraviolet irradiation on a number of DNA genome viruses and appears to inhibit DNA repair mechanisms (4, 19, 29), and also seems to affect infectivity and ultraviolet light induction of simian virus 40 (20, 27). It has been suggested that caffeine may even bind to DNA (5, 10, 22, 23). The survival of double-stranded, replicative form RNA from encephalomyocarditis virus after UV-irradiation has been shown to decrease if caffeine is added to the growth medium thus indicating a possible host cell repair mechanism for RNA (28).

Since caffeine has been demonstrated to have a profound effect on the biochemistry of DNA and of RNA survival, it was of interest to examine the direct effects of caffeine on an RNA genome virus. Newcastle disease virus (NDV), an enveloped paramyxovirus with a helical nucleocapsid was selected for our studies.

This report describes that caffeine affects NDV synthesis by altering the virion polypeptide composition of the viral progeny produced. The Caf+ virions were found to be ultrasensitive to common laboratory treatment when compared to untreated virus (Caf-).

MATERIALS AND METHODS

Virus, Cells, and Medium. The Roakin strain of NDV was used in all experiments. Chicken embryo cell cultures were made from 10 to 12-day-old embryonating chicken eggs as previously described (16). Cultures were grown to complete monolayers in Eagle's MEM (6) containing 5% fetal calf serum. After adsorption of virus (20 plaque-forming units (PFU)/cell) for 30 min, the cultures were maintained in Eagle's MEM with (Caf+) or without (Caf-) caffeine and without serum. These abbreviations (Caf+, Caf-) will be used throughout the manuscript to indicate infected or noninfected cells maintained in medium with (Caf+) and without (Caf-) caffeine or to specify purified virus preparations obtained from both kinds of cultures.

Caffeine. The caffeine (Eastman Kodak Co., Rochester, N. Y.) was dissolved in 0.1 ml ethanol and diluted with Eagle's MEM (without serum) to a concentration of 50 mM, filter sterilized, and stored at -20 C. When used, this stock caffeine was diluted to 10 mM with medium.

Virus purification and titration. Infected cultures were maintained in medium with or without caffeine. Three times as many caffeine-treated, infected cell cultures than untreated, infected cultures were used in the NDV purification studies to obtain comparable virus titers (Caf+, Caf-). For continuous radioactive labeling of virus, the medium was supplemented with 5 μCi ^{3}H-uridine/ml.

Cultures were harvested 36 h after infection, and cells were disrupted in a Sorvall omnimixer (Ivan Sorvall Inc., Norwalk, CT) at maximal rheostat setting for 5 min at 4 C. The homogenate was treated with 10 units/ml receptor-destroying enzyme (Calbiochem Corp., Los Angeles, CA) for 4 h at ambient temperature. Cell debris was removed by centrifugation at 10,000 rpm

for 30 min, and NDV was concentrated by pelleting through a 20% neutral sucrose shelf at 21,000 rpm (SW27 rotor) for 2 h. The pelleted virus was resuspended and pelleted through another 20% neutral sucrose shelf at 30,000 rpm (SW50.1 rotor) for 1.5 h. Resuspended virus (TE buffer, 0.002 M Tris and 0.002 M EDTA, pH 7.4) was banded through a 30-50% continuous glycerol-potassium tartrate gradient (17) at 35,000 rpm for 10 h. Fractions with coincident peaks of radioactivity and hemagglutination activity were pooled and dialyzed against TE buffer.

The NDV was quantitated by plaque and hemagglutination assay as previously described (16). Virus buoyant density was determined by weighing samples of the glycerol-potassium tartrate gradient fractions.

Antigen synthesis. Synthesis of structural protein(s) of NDV was assayed by the indirect immunofluorescent technique (9). Cells were examined for cytoplasmic fluorescence using a Leitz orthoflux fluorescent microscope. Synthesis of hemagglutinin in NDV-infected cells was quantitatively determined by the method of Finter (7).

Incorporation of radioisotopic precursors. Protein synthesis was determined by exposing infected and noninfected cultures (Caf+, Caf-) to 5 μCi ^{3}H-valine/ml. The isotopic incorporation was terminated at various intervals by adding cold 5% trichloroacetic acid (TCA) to the monolayer. Cells were washed 3 times with 5% TCA to remove unincorporated label, and the cells were hydrolyzed with hot 1N NaOH and assayed for incorporation of radioactive precursor and protein content.

Viral RNA synthesis was determined in infected chicken embryo cells maintained in Eagle's MEM (Caf+, Caf-) supplemented with 5 μg actinomycin D/ml (Merck and Company, Rahway, NJ) and 5 μCi ^{3}H-uridine/ml. Incorporation

of isotopic precursor into RNA was terminated at various intervals with cold 5% TCA. Monolayers were washed 3 additional times with 5% TCA, and the cells were hydrolyzed in hot 1N NaOH and assayed for incorporation of radioactive precursor and protein content.

Radioactivity was measured in toluene-triton X-100 (3:1) scintillation fluid using a Beckman LS233 liquid scintillation counter. Total protein was determined by the Folin-phenol method (12), using bovine serum albumin as the standard.

Sensitivity of NDV to ultraviolet irradiation. Equivalent titers of plaque forming units (PFU) of purified NDV from infected cultures (Caf+, Caf-) were diluted in phosphate-buffered saline (PBS: 0.001 M PO_4, pH 7.4; 0.15 M NaCl) and distributed in 2 ml amounts to 35 mm plastic petri dishes. The uncovered dishes were placed in a UV chamber and irradiated by a General Electric 15 Watt germicidal lamp at a distance of 54 cm using a slit width of 15 cm. Stability of the purified viral preparations to UV-irradiation was determined by plaque assay.

Sensitivity of NDV to freeze-thaw. Equivalent titers (PFU) of purified NDV from infected cultures (Caf+, Caf-) were diluted in TE buffer, distributed to glass vials, and subjected to various cycles of freeze-thaw. The viral preparations were held in the frozen state at -20 C and quick-thawed in a water bath at 37 C. After each cycle, the preparations were vortexed for 5 s, a sample was withdrawn from each (Caf+, Caf-), and the stability of the viral preparations to freezing and thawing was determined by plaque assay.

Effect of freezing and thawing on purified virions. NDV was purified (as described above) from infected cultures (Caf+, Caf-) that were maintained

in medium containing 5 μCi ^{3}H-uridine/ml. From the respective gradients, fractions with coincident peaks of radioactivity and hemagglutination activity were pooled and dialyzed against TE buffer to remove the glycerol-tartrate. The purified NDV preparations (Caf+, Caf-) were each equally divided into two aliquots. One aliquot of each of the preparations (Caf+, Caf-) was subjected to one cycle of freeze-thaw and the remaining two aliquots (Caf+, Caf-) were untreated. The four samples were then centrifuged in glycerol-tartrate gradients as described above. Fractions were collected and assayed for radioactivity and hemagglutination activity.

SDS-Polyacrylamide gel electrophoresis. Forty micrograms of purified NDV (Caf+, Caf-) were precipitated by the addition of cold TCA to a final concentration of 25%. After 2 h at 0 C, the precipitates were collected by centrifugation at 10,000 rpm (Sorvall HB-4 rotor) for 30 min, and the precipitates were washed twice with cold acetone and air dried. Each precipitate (Caf+, Caf-) was resuspended in phosphate buffer (10^{-3} M, pH 7.5). The disruption of the proteins in the SDS-β-mercaptoethanol mixture has been described (13). The proteins were electrophoresed through a 5% stacking gel and 12.5% running slab gel. The buffer contained 0.1% SDS, 0.38 M glycine in Tris buffer (0.05 M, pH 8.4). Equivalent protein concentrations of each virus sample (Caf+, Caf-) were loaded onto the gel, and 15 mA/gel was applied for 30 min until the protein had stacked at the gel interface. The current was then increased to 30 mA/gel until the tracking dye had reached the bottom of the gel. The gel was fixed and stained in 50% methanol-7.5% acetic acid containing 0.1% Coomassie blue. The gel was destained by repeated washes of 5% methanol-7.5% acetic acid. The wet gel was photographed with Kodak Film Type 55-PN. The negative was used to make a densitometry scan with a Joyce Loebl Recording Densitometer which allowed

quantitation of the individual, viral polypeptides. The area under each peak of the polypeptide scan was measured, and the values were used to determine the amount of each polypeptide as a percent of the total protein. Molecular weights were determined by the method of Weber and Osborn (24).

RESULTS

Effect of caffeine on the synthesis of NDV. We were interested in examining the effects of caffeine on NDV replication. For studies of this nature, it is essential to use a drug concentration that produces the maximum effect on the virus with minimum damage to the host cells. Caffeine concentrations ranging from 1.0 mM to 10 mM were added to the maintenance medium, and a maximum 84% reduction in viral progeny synthesis was observed using 10 mM caffeine (Table 1). Therefore, 10 mM caffeine was selected as the desired concentration for future experiments. This inhibition was not a reflection of host cell death, since noninfected cultures could be maintained in 10 mM caffeine-supplemented medium for 24 h without the appearance of cytotoxic effects.

An 18 h growth experiment was performed to compare viral replication completed under Caf+ and Caf- conditions. Infected cultures were harvested at various intervals after infection, and the cultures were plaque-assayed for infective viral synthesis. In the Caf+ medium, viral synthesis was inhibited 95% when compared to infected cultures that were maintained in Caf- medium (Fig. 1).

Viral RNA synthesis. After establishing the inhibitory effect of caffeine on viral replication, studies were performed to examine the effect of the drug on synthesis of viral constituents during replication.

Chicken embryo cells were infected with NDV and maintained in medium (Caf+, Caf-) with added actinomycin D and ^{3}H-uridine. The amount of radioactivity incorporated into the TCA-insoluble material was determined every 2 h after infection. As shown in Figure 2, incorporation of ^{3}H-uridine into noninfected cells was not observed. However, at 12 h

postinfection, ^{3}H-uridine incorporation in infected cells was 63% less in Caf+ cells than in Caf- cells. Since actinomycin D-resistant incorporation of ^{3}H-uridine is considered to represent the synthesis of viral RNA (11), it may be concluded that the synthesis of NDV-specific RNA is inhibited by caffeine treatment.

Protein synthesis. The effects of caffeine on cellular protein synthesis were examined (1) by following the incorporation of labeled ^{3}H-valine into acid-insoluble protein, (2) by following the synthesis of intracellular viral hemagglutinin, and (3) by synthesis of NDV structural proteins determined by immunofluorescence.

Caffeine addition decreased the incorporation of ^{3}H-valine in infected cultures by 45% after 10 h of infection when compared with cultures maintained without caffeine (Fig. 3). Infected cells maintained in Caf+ or Caf- medium were assayed for hemadsorption ability. In infected cells maintained in Caf+ medium, a 25% reduction in hemadsorption ability was observed when compared to that found in Caf-, infected cells (Fig. 4).

To eliminate the possibility that fewer cells were being infected in the Caf+ cultures, indirect immunofluorescence was used to determine synthesis of NDV structural antigens. The number of cells synthesizing NDV structural antigens was identical in both Caf+ and Caf- cultures (Table 2). However, the infected cultures maintained in Caf+ medium had reduced fluorescence within individual cells indicating less protein antigen(s) was being synthesized per infected cell. Each of these data indicates that caffeine is indeed inhibiting synthesis of viral specific proteins.

Viral purification. Having observed differences produced by caffeine on the synthesis of NDV protein(s) and RNA, it was of interest to determine

if the drug affected NDV assembly. NDV obtained from infected cultures (Caf+, Caf-) supplemented with ^{3}H-uridine were purified by centrifugation in glycerol-tartrate gradients (Fig. 5). Although a major and a minor peak of radioactivity appeared in both the Caf+ and Caf- samples, assays showed that hemagglutinating ability and plaque-forming ability corresponded only with the material under the major peaks. The density of the material under the major peak was determined to be 1.13 g/cm^{3} for the virus from Caf+ cultures and 1.17 g/cm^{3} for the virus from Caf- cultures. Purified virus from Caf+ cultures were consistently less dense than NDV from untreated cultures.

Stability of purified Caf+ and Caf- NDV. Identical titers (PFU) of both purified NDV preparations (Caf+, Caf-) were exposed to low doses of ultraviolet irradiation to determine virion sensitivity to irradiation. Figure 6 shows that the Caf+ virions experienced an immediate 15% decrease in survival after 2 s of irradiation and a 40% decrease by 6 s of exposure while Caf- virions were still unaffected. After 9 s of irradiation, the survival rate was 50% for the Caf+ virions and 70% for the Caf- virions.

Purified NDV was also examined for sensitivity to various cycles of freeze-thaw. Identical titers (PFU) of both purified virion preparations (Caf+, Caf-) were alternately frozen and thawed five consecutive times. After each cycle of freeze-thaw, a sample of each preparation was assayed for infectivity (PFU). Figure 7 illustrates that after one cycle of freeze-thaw, 65% of the Caf+ virions were inactivated as compared to 10% inactivation for the Caf- virions. After five cycles of freeze-thaw, survival was reduced to 15% in the Caf+ virion preparation and to only 55% in the Caf- virions.

An additional experiment was performed to examine the extreme lability of the purified Caf+ virions to one cycle of freeze-thaw and to determine the effect of this treatment on virion stability. NDV was purified from infected cultures that were maintained in medium (Caf+, Caf-) supplemented with ^{3}H-uridine. One aliquot of the purified Caf+, ^{3}H-NDV and one aliquot of the Caf-, ^{3}H-NDV was subjected to one cycle of freeze-thaw, while one aliquot of each purified virus preparation was not subjected to the harsh treatment. The four samples were then centrifuged on separate glycerol-tartrate gradients. When the gradients were collected, each was shown to contain a major peak of radioactivity coincident with hemagglutination activity. There was radioactivity with no hemagglutination activity at the top of each gradient which consisted of virion breakdown products (Fig. 8A and B). Both freeze-thawed virion (Caf+, Caf-) preparations experienced greater reductions in their virion peaks than was seen in their unfrozen counterpart preparations. This reduction in the virion peak was accompanied by an increased amount of radioactive virion breakdown products at the top of the gradients. However, the Caf+ virion preparation that was exposed to freezing and thawing had a 75% reduction in virion stability (peak) when compared to 50% reduction seen in the frozen and thawed Caf- preparation. It should be noted that a small reduction in virion stability was seen even in the unfrozen virion preparations (Caf+, Caf-). This was probably due to the sedimentation forces of centrifugation, which could also have enhanced viral lability caused by freezing and thawing.

NDV polypeptides. Due to the observed differences in virion (Caf+, Caf-) stability, it was decided to compare the polypeptides of purified NDV in order to determine if virion composition may be responsible for virion

instability. Figure 9 illustrates a typical densitometer pattern from respective SDS-polyacrylamide gels obtained for the polypeptides of NDV. Polypeptide analysis demonstrated that the NDV molecular weight determinations were in close agreement with those previously reported for NDV (14). Identical polypeptide patterns were found in both purified virion (Caf+, Caf-) preparations, but the relative amounts of several of the polypeptides appeared to be altered (Table 3). Caf+ virions demonstrated an increase in the F_o,F (precursor to fusion glycoprotein; fusion and hemolysis glycoprotein) and HN (hemagglutinating and neuraminidase glycoprotein) proteins and a reduction in the S, M (membrane protein), and L proteins. The L protein has been previously reported in paramyxovirus and rhabdovirus and may be involved in transcriptase activity (8). The remaining C and NP (nucleocapsid) proteins appear to contain approximately the same amount of polypeptide in both Caf+ and Caf- virions.

DISCUSSION

The data presented in this report demonstrate that caffeine produces an inhibitory effect on an NDV infection. Addition of caffeine to growth medium caused reduction in RNA synthesis (Fig. 2), in protein synthesis (Fig. 3), in hemagglutinin synthesis (Fig. 4), and reduction in progeny synthesis (Fig. 1) in NDV infected cultures when compared to untreated, infected cultures. The fluorescent antibody study showed this reduction was not due to fewer cells being infected, since an equivalent number of cells showed fluorescence in both treated and untreated cultures (Table 2). Purified virions obtained from Caf+ cultures were lighter in density, and appeared to be more sensitive to ultraviolet light irradiation (Fig. 6) and to damage by freeze-thaw (Fig. 7). Caf+ virions also contained a complement of structural proteins which differed in relative amounts (Fig. 9 and Table 3).

The extreme sensitivity of Caf+ virions to freezing and thawing indicated that these virions may have altered structural proteins which caused an inherent weakness within the virus structure. It was found that several polypeptides appear to differ in relative proportions in the Caf+ virus when compared to the Caf- virus. The major differences appear to be a decreased amount of M and S protein and an increased amount of F_o,F protein in the Caf+ virions. The exact effect each alteration in protein quantity may have on virion stability is unknown at this time. The increased quantity of F_o,F protein found in Caf+ virions may have been caused because caffeine could have prevented the proteolytic cleavage of the precursor (F_o) glycoprotein. It appears well established in other paramyxoviruses that cleavage of a precursor of the virion glycoprotein is required for activation

of cell fusion, hemolysis, and infectivity, and that whether such cleavage occurs depends on the host cell (1). Also, a small molecular weight protein (S) has been identified as the cleavage product from the precursor to fusion glycoprotein in measles virus (Marie Hardwick, manuscript in press, J. Virology) and in SV5 (18). If the S protein of NDV is a similar cleavage product of the precursor to fusion glycoprotein (F_o), the reduced amount of S protein which accompanied the increased F_o,F protein in the Caf+ virus may support the suggestion that cleavage of F_o to F was inhibited. In studies with influenza virus, the M protein appears to be responsible for maintaining the stability of the intact virion (2). Compared with influenza virus, relatively little is known about the minor polypeptides of paramyxoviruses. However, it is known that the M protein is a nonglycosylated protein which appears to be associated with the inner surface of the paramyxovirus envelope (1, 21, 26). Studies with SV5 (15) suggest a possibile affinity between the nucleocapsid and the membrane protein. This indicates the M protein may be involved with virus assembly (1) since, during influenza and parainfluenza maturation, nucleocapsids appear to interact only with regions of the cell membrane which contain viral specific proteins (2, 3).

There also appeared to be an increase in the amount of HN protein in Caf+ virions, although hemagglutinin synthesis was reduced by 25% (Fig. 4) in infected cells maintained in Caf+ medium. However, it should be noted that data from the protein analysis of NDV represent protein quantities from assembled, purified virions, while the hemagglutinin data was obtained from infected cell cultures.

In conclusion, it is our belief that caffeine produces an inhibitory effect on NDV synthesis by possibly altering the expression of the viral

genome culminating in an alteration of the viral proteins. This alteration could affect the regulation of protein incorporation into the cell membrane which is eventually programmed to become the virion envelope. These differences in protein amounts or arrangement within the envelope during viral maturation may result in increased virus lability. Apparently, caffeine has altered the protein composition of the virus, but whether this effect on NDV proteins is at the level of transcription or translation is yet to be determined.

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Figure 1. Effect of caffeine on Newcastle disease viral synthesis in chicken embryo cells. ●——● = Infected cultures maintained in Eagle's medium; O——O = infected cultures maintained in Eagle's medium with caffeine.

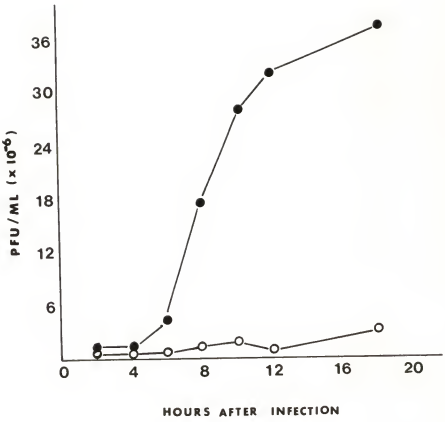
36
30
24
18
12
6
PFU/ML (x 10⁻⁶)
0
4
8
12
16
20
HOURS AFTER INFECTION

Figure 2. Effect of caffeine on the incorporation of ^{3}H-uridine into acid insoluble material in NDV infected chicken embryo cells. Non-infected and infected chicken embryo cells were incubated in the presence of ^{3}H-uridine and actinomycin D. The radioisotope incorporated into acid-insoluble material was determined. ●——● = infected cultures maintained in Eagle's medium; ▲——▲ = infected cultures maintained in Eagle's medium with caffeine; O——O = noninfected cultures maintained in Eagle's medium; △——△ = noninfected cultures maintained in Eagle's medium with caffeine.

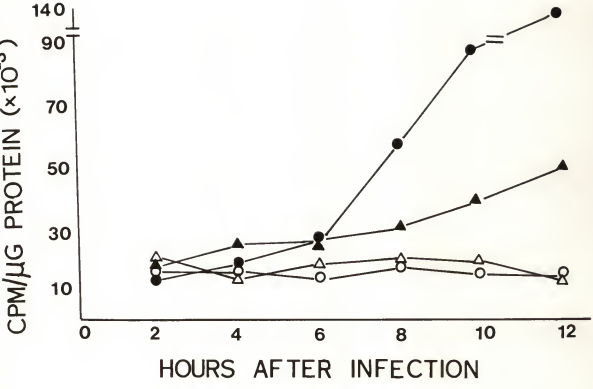
140
90
70
50
30
10
CPM/µG PROTEIN (×10⁻³)
0
2
4
6
8
10
12
HOURS AFTER INFECTION

Figure 3. Effect of caffeine on the incorporation of ^{3}H-valine into acid-insoluble material in NDV-infected chicken embryo cells. ●——● = infected cultures maintained in Eagle's medium; ▲——▲ = infected cultures maintained in Eagle's medium with caffeine; O——O = non-infected cultures maintained in Eagle's medium; △——△ = noninfected cultures maintained in Eagle's medium with caffeine.

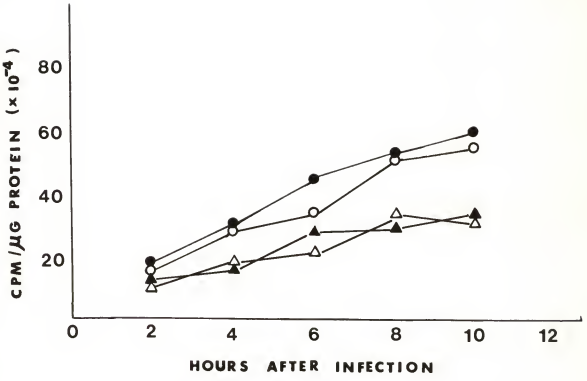
CPM/μG PROTEIN (×10⁻⁴)
80
60
40
20
0
2
4
6
8
10
12
HOURS AFTER INFECTION

Figure 4. Effect of caffeine on hemagglutinin synthesis in NDV-infected chicken embryo cells as determined by hemadsorption. ●——● = infected cultures maintained in Eagle's medium; O——O = infected cultures maintained in Eagle's medium with caffeine; ▲——▲ = noninfected cultures maintained in Eagle's medium; △——△ = noninfected cultures maintained in Eagle's medium with caffeine.

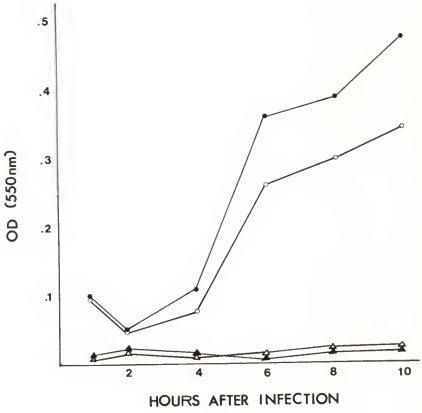
.5
.4
.3
.2
.1
OD (550mm)
2
4
6
8
10
HOURS AFTER INFECTION

Figure 5. Purification of NDV. The partially purified virus was further purified on a 30 to 50% continuous glycerol-potassium tartrate gradient and centrifuged at 35,000 rpm for 10 h. Fractions from the (A) Caf+ virus and (B) Caf- virus gradients were collected and assayed. O———O = hemagglutination activity; ●———● = radioisotopic incorporation; ▲———▲ = buoyant density.

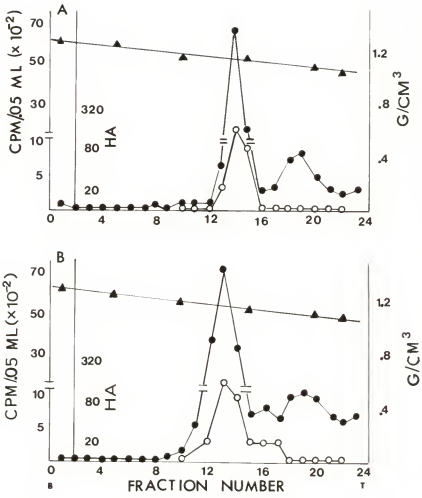
A
B
CPM/.05 ML (x 10⁻²)
70
50
30
10
5
320
80
20
HA
1.2
.8
.4
G/CM³
0
4
8
12
16
20
24
FRACTION NUMBER

Figure 6. Effect of ultraviolet irradiation on purified NDV. ●——● = purified NDV from infected cultures maintained in Eagle's medium; O——O = purified NDV from infected cultures maintained in Eagle's medium with caffeine.

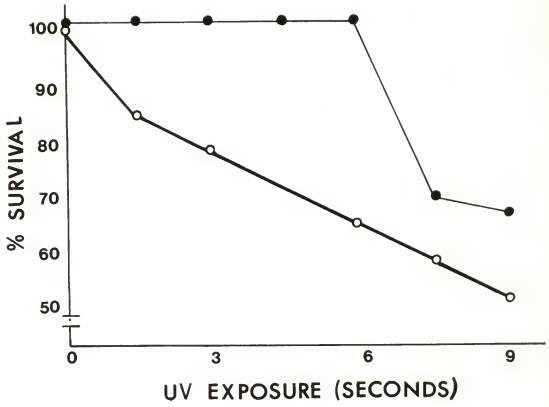
100
90
80
70
60
50
% SURVIVAL
0
3
6
9
UV EXPOSURE (SECONDS)

Figure 7. Effect of freezing and thawing on purified NDV. ●——● = purified NDV from infected cultures maintained in Eagle's medium; O——O = purified NDV from infected cultures maintained in Eagle's medium with caffeine.

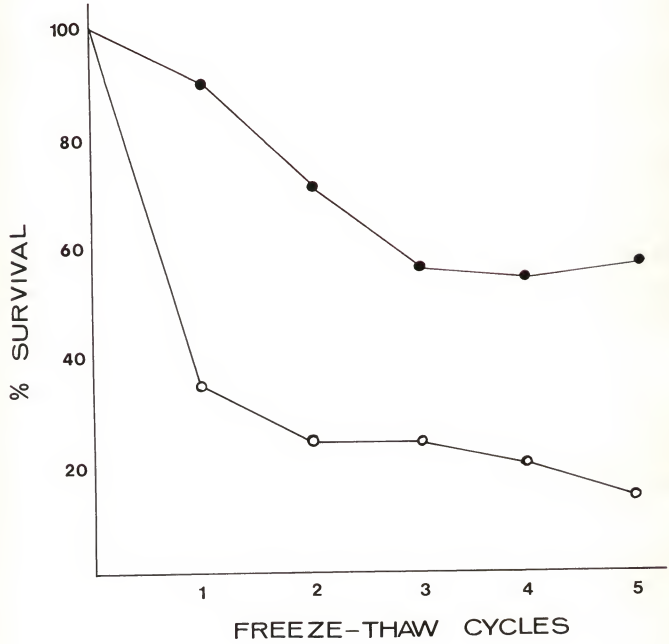
100
80
60
40
20
% SURVIVAL
1
2
3
4
5
FREEZE-THAW CYCLES

Figure 8. Effect of freezing and thawing on purified NDV. Purified virions (Caf+, Caf-) were subjected to a single freeze-thaw and centrifuged on glycerol-tartrate gradients for 10 h at 35,000 rpm. Fractions from the (A) Caf+ virus and (B) Caf- virus gradients were collected and assayed for radioactivity. ●———● = purified NDV which was not freeze-thawed; O———O = purified NDV which was exposed to one freeze-thaw.

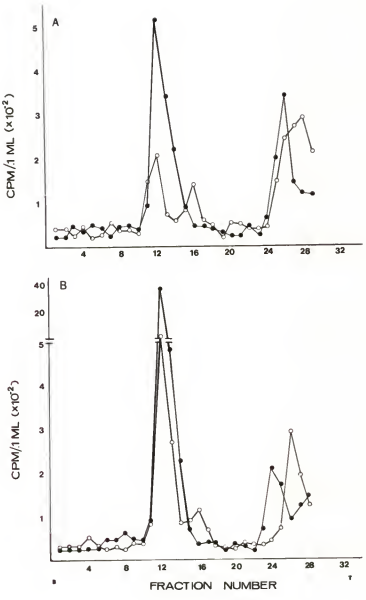
A
B
CPM/1 ML (x10²)
CPM/1 ML (x10²)
FRACTION NUMBER
4
8
12
16
20
24
28
32
1
2
3
4
5
20
40

Figure 9. Densitometry tracing of SDS-polyacrylamide gel of NDV polypeptides. Starting from the left, the molecular weight markers indicated by the arrows are Cytochrome C (12,000), α-Chymotrypsinogen (26,000), Ovalbumin (44,000), and Bovine Serum Albumin (68,000). (———) = proteins of purified NDV from infected cultures maintained in Eagle's medium; (-----) = proteins of purified NDV from infected cultures maintained in Eagle's medium with caffeine.

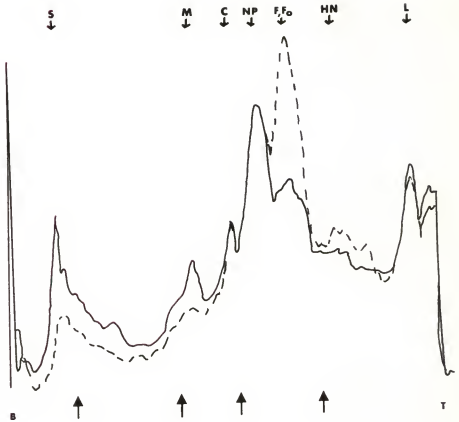
S
M
C
NP
F,Fo
HN
L
B
T

TABLE 1. Caffeine inhibition of NDV synthesis

CAFFEINE CONCENTRATION	PFU[a]	% INHIBITION
0.0 mM	7.0×10^7	--
1.0 mM	3.8×10^7	46
2.5 mM	2.5×10^7	65
5.0 mM	1.7×10^7	76
10.0 mM	1.2×10^7	84

[a]Infected cultures were assayed for plaque forming units (PFU) at 12 hours post-infection.

TABLE 2. Effect of Caffeine on Synthesis of NDV-Specific Antigen(s)[a]

	Percentage of Positive Cells[b]	
Hours after Infection	Complete Medium	Complete Medium with Caffeine
0	1	1
10	91	90

[a]Synthesis of viral antigen(s) was determined by indirect immunofluorescence.

[b]Percentage was based on 300 cells counted.

TABLE 3. NDV Polypeptide Analysis

Polypeptide	Mol. Wt.	% of Total Protein Caf+	Caf-
L	130,000	16.6	18.4
HN	75,000	10.6	8.8
F_o F	59,000	33.2	22.4
NP	49,000	24.4	24.8
C	41,000	6.6	6.3
M	32,000	4.2	7.8
S	12,000	4.4	11.5

Percentages were determined by calculations of the area under each peak as seen in Fig. 9.

INHIBITION OF NEWCASTLE DISEASE VIRUS INFECTION OF CHICKEN EMBRYO CELLS BY CAFFEINE

by

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B.A., Bethany College, 1975

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ABSTRACT

Newcastle disease is a respiratory disease of chickens of considerable importance to the United States poultry industry. For this reason, much research has been accomplished to determine the in vivo and in vitro effects caused by Newcastle disease virus (NDV) infection in an effort to understand the mechanism(s) involved.

NDV, a paramyxovirus, is a roughly spherical, enveloped virus containing single-stranded ribonucleic acid (RNA). Some paramyxoviruses are known to infect humans. NDV is an ideal model system for the study of these viruses. The use of drugs to study paramyxoviruses may give information concerning growth control mechanisms. It is hoped that one day this information may lead to the development of viral specific drugs which have minimum toxic effects on the virus host cells. Also, there is always the possibility that information gained may help in understanding cellular processes in general.

Caffeine is a drug that has been used in studies involving prokaryotic and eukaryotic cell systems. Some investigators have utilized caffeine to examine UV survival and induction processes of DNA viruses. However, minimal investigation has been done with RNA viruses. The purpose of this investigation was to describe the effects of caffeine on NDV synthesis in cultured chick embryo cells, and to elucidate a possible mechanism for the effects observed.

Addition of 10 mM caffeine to growth medium resulted in a 63% reduction in RNA synthesis, a 45% reduction in protein synthesis, a 25% reduction in

hemadsorption ability and a 95% reduction in progeny synthesis in NDV infected cultures when compared to untreated, infected cultures. Purified virions from caffeine-treated cultures were lighter in density and appeared to be more sensitive to ultraviolet light irradiation and to damage by freeze-thaw. When the virus structural proteins were examined, caffeine-treated virions had an increased amount of F_o,F protein and a reduced amount of M and S protein when compared to untreated, purified NDV. Thus, caffeine appears to alter the protein composition of the virus which may result in increased virus lability.